# Water deficit induces chlorophyll degradation via the 'PAO/phyllobilin' pathway in leaves of homoio- (Craterostigma pumilum) and poikilochlorophyllous (Xerophyta viscosa) resurrection plants

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# Water deficit induces chlorophyll degradation via the "PAO/phyllobilin" pathway in leaves of homoio- (*Craterostigma pumilum*) and poikilochlorophyllous (*Xerophyta viscosa*) resurrection plants

Running title: Chlorophyll breakdown in resurrection plants

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## ABSTRACT

Angiosperm resurrection plants exhibit poikilo- or homoiochlorophylly as a response to water deficit. Both strategies are generally considered as effective mechanisms to reduce oxidative stress associated with photosynthetic activity under water deficiency. The mechanism of water deficit-induced Chl degradation in resurrection plants is unknown but has previously been suggested to occur as a result of nonenzymatic photooxidation. We investigated Chl degradation during dehydration in both poikilochlorophyllous (*Xerophyta viscosa*) and homoiochlorophyllous (*Craterostigma pumilum*) species. We demonstrate an increase in the abundance of PHEOPHORBIDE *a* OXYGENASE (PAO), a key enzyme of Chl breakdown, together with an accumulation of phyllobilins, i.e. products of PAO-dependent Chl breakdown, in both species. Phyllobilins and PAO levels diminished again in leaves from rehydrated plants. We conclude that water-deficit induced poikilochlorophylly occurs via the well characterized PAO/phyllobilin pathway of Chl breakdown and that this mechanism also appears conserved in a resurrection species displaying homoiochlorophylly. The role(s) of the PAO/phyllobilin pathway during different plant developmental processes that involve Chl breakdown, such as leaf senescence and desiccation, fruit ripening and seed maturation, are discussed.



## KEYWORDS

chlorophyll breakdown, *Craterostigma pumilum*, desiccation tolerance, homoiochlorophyllous, PAO/phyllobilin pathway, phyllobilins, poikilochlorophyllous, resurrection plants, *Xerophyta viscosa*

## INTRODUCTION

In contrast to the majority of plants, resurrection or desiccation-tolerant plants are characterized by their ability to tolerate and survive extreme desiccation, withstanding the loss of up to 90-95% of the cellular water in their vegetative tissues and resuming normal metabolism within a short period after rehydration (Gaff & McGregor 1979; Farrant 2000; Scott 2000; Proctor & Tuba 2002; Vicré, Farrant & Driouich 2004). Desiccation tolerance entails cellular, biochemical, and molecular changes during dehydration (Vicré *et al.* 2004), including the synthesis of (i) carbohydrates (Whittaker *et al*. 2001; Peters et at., 2007; Toldi, Tuba & Scott 2009), (ii) intrinsically disordered proteins such as late embryogenesis-abundant proteins and small heat shock proteins (Ingram & Bartels 1996), (iii) antioxidants (Kranner *et al*. 2002; Mowla *et al*. 2002; Vicré *et al*. 2004) and, (iv) volatile and non-volatile isoprenoids (Beckett *et al.* 2012).

Resurrection plants are grouped into two categories regarding chlorophyll (Chl): homoiochlorophyllous and poikilochlorophyllous plants. Homoiochlorophyllous species retain most or all Chl during drying and preserve their photosynthetic apparatus, while poikilochlorophyllous species lose most of their Chl and dismantle the photosynthetic apparatus during dehydration (Tuba *et al*. 1994; Sherwin & Farrant 1996; Tuba, Proctor & Csintalan 1998). Parallel to the loss of Chl, chloroplasts of the poikilochlorophyllous genera *Xerophyta* show grana dismantling, thylakoid vesiculation, and cessation of photosynthetic $CO_2$ assimilation and respiration (Gaff & McGregor 1979; Tuba *et al*. 1996; Collett *et al*. 2003; Ingle *et al*. 2008). Chl breakdown during desiccation seems to be related to protection against oxidative damage caused by overreduction of the electron transport chain (Farrant 2000). This hypothesis is supported by the observation that *Xerophyta scabrida* preserves most of the Chl when dried in the dark, but degrades Chl during desiccation under day/night growth conditions (Tuba *et al*. 1996). Upon rehydration, photochemical activity recovers rapidly in *Xerophyta* species (Farrant 2000). Transition of desiccoplasts, i.e. plastids of dehydrated poikilochlorophyllous leaves (also termed xeroplasts in *Xerophyta* species) to photosynthetically active chloroplasts implies formation of grana, Chl biosynthesis, and light-dependent restoration of photosystem (PS) II (PSII) photochemistry and $CO_2$ assimilation (Ingle *et al*. 2008; Pérez *et al*. 2011; Solymosi, Tuba & Böddi 2013).

By contrast, during desiccation in homoiochlorophyllous plants (like the genera *Craterostigma*), chloroplasts adopt an oval shape and partially lose their internal organization with grana stacks largely preserved (Schneider *et al*. 1993). Active protection mechanisms of chloroplasts in homoiochlorophyllous plants also include the transient accumulation of plastid-localized stress proteins (Schneider *et al*. 1993; Gechev *et al*. 2012). It has been argued that a combination of physical (inward curling of leaves) and chemical (increase in anthocyanin content and antioxidative enzyme activities) mechanisms enable protection against potential photooxidative damage resulting from Chl excitation in dry leaves (Sherwin & Farrant 1998).

To date, the mechanism of Chl degradation during desiccation in resurrection plants is unknown. In poikilochlorophyllous species, Chl degradation has been suggested to be the result of photooxidation rather than of enzymatic processes (Tuba *et al*. 1996; Proctor & Tuba 2002). Here, we investigated whether Chl degradation during desiccation in the poikilochlorophyllous species *Xerophyta viscosa* (*X. viscosa*) and the homoiochlorophyllous species *Craterostigma pumilum* (*C. pumilum*) follows the well-described "PAO/phyllobilin pathway", shown to be active during leaf senescence and fruit ripening of higher plants (reviewed in Kräutler 2008; Hörtensteiner & Kräutler 2011; Christ & Hörtensteiner 2013; Kräutler & Hörtensteiner 2013). In this pathway (Fig. 1), Chl is ultimately broken down to a group of linear tetrapyrroles, termed phyllobilins (Kräutler & Hörtensteiner 2013). The major classes of phyllobilins identified from different species can be categorized into formyloxobilin-type nonfluorescent Chl catabolites (NCCs) (Kräutler & Matile 1999) and dioxobilin-type nonfluorescent Chl catabolites (DNCCs) (Christ *et al*. 2013; Süssenbacher *et al*. 2014; Kräutler & Hörtensteiner 2013). The structural relation of these phyllobilins is based on the activity of PHEOPHORBIDE *a* OXYGENASE (PAO), a key enzyme of the pathway (Hörtensteiner *et al*. 1998), which together with RED CHLOROPHYLL CATABOLITE REDUCTASE (RCCR) (Rodoni *et al*. 1997) catalyzes the oxygenolytic porphyrin ring opening of pheophorbide *a*, a phytol- and Mg-free intermediate of breakdown, yielding a *primary* fluorescent Chl catabolite (*p*FCC) (Mühlecker *et al*. 1997; Pružinská *et al*. 2007) or its epimer, *epi-p*FCC (Mühlecker *et al*. 2000) (Fig. 1). It was recently shown in *Arabidopsis thaliana* that protein-protein interaction between the chloroplast-localized enzymes of the PAO/phyllobilin pathway allows metabolite channeling between

Chl and *p*FCC and, thus, controlled degradation of potentially phototoxic Chl catabolites (Sakuraba *et al*. 2012; Sakuraba *et al*. 2013). *p*FCC, considered to be the first non-phototoxic Chl catabolite, is exported from the chloroplast (Hörtensteiner 2006). Subsequently, modified fluorescent Chl catabolites are formed in the cytosol by species-specific functionalization of *p*FCC at different peripheral side groups. After import into the vacuole, these fluorescent compounds are converted to respective NCCs through isomerization catalyzed by the acidic vacuolar pH (Oberhuber *et al*. 2003; Kräutler & Hörtensteiner 2013). NCCs have been shown to be prone to further oxidation (Ulrich *et al*. 2011) yielding yellow Chl catabolites (YCCs). Chemical oxidation of NCCs to YCCs has been demonstrated in vitro, however, YCCs were also found in extracts of fresh senescent leaves of *Cercidiphyllum japonicum*, indicating that they may also be formed in vivo (Moser *et al*. 2008; Ulrich *et al*. 2011; Scherl, Müller & Kräutler 2012) (Fig. 1).

To date, Chl catabolites (Kräutler 2008; Kräutler & Hörtensteiner 2013) and breakdown mechanisms have largely been described during senescence processes, which ultimately lead to the death of respective tissues (Hörtensteiner 2006; Hörtensteiner & Kräutler 2011). Here, we provide evidence that Chl breakdown during desiccation of the poikilochlorophyllous resurrection plant *X. viscosa* is mediated by the PAO/phyllobilin pathway (Kräutler & Hörtensteiner 2013). Thus, like during leaf senescence (Pružinská *et al*. 2005), PAO is up-regulated and several NCCs accumulate during the drying phase in *X. viscosa* leaves. Upon rehydration, NCC abundance decreases in regreening leaves. In addition, our study indicates that Chl breakdown also follows the PAO/phyllobilin pathway and abundance of Chl catabolites decrease during rehydration in the homoiochlorophyllous species *C. pumilum*. However, compared to *X. viscosa*, significant amounts of YCCs accumulate, indicating higher oxidative damage in the homoiochlorophyllous species.

## MATERIALS AND METHODS

### Plant material

*X. viscosa* and *C. pumilum* plants were propagated under greenhouse conditions as previously described (Bartels *et al*. 1990; Peters *et al*. 2007). Plants used for water deficit experiments were

grown in a controlled-environment chamber (*X. viscosa*, 16 h light with 130 µmol photons $m^{-2}$ $s^{-1}$ / 8 h dark, 25°C, 60% relative humidity; *C. pumilum*, 14 h light with 180 µmol photons $m^{-2}$ $s^{-1}$, 24°C / 10 h dark, 20°C, 60% relative humidity).

**Water deficit stress treatment**

Water deficit stress was imposed on whole potted plants by withholding irrigation at the end of which the relative water content (RWC) was determined to be 5-10%. Leaf samples were excised at regular intervals, immediately flash-frozen in liquid nitrogen, ground, freeze-dried and stored at -80°C. Sampling times were determined by visual appraisal of the plants using leaf decoloration and folding as benchmarks, at which times the RWC of leaves was determined as previously described (Barrs & Weatherley 1962; Peters *et al*. 2007). Rehydration was conducted by watering the plants and sampling as described above. For *C. pumilum*, "non-senescent" (surviving) and "senescent" (yellowing and dying) leaf samples were collected separately at four different time points ["hydrated" (RWC: ~80-95%), "partially dehydrated" (RWC: ~30-40%), "dried" (RWC: ~5-10%) and "rehydrated" (RWC: ~80-95%)]. Images of leaf sections (*X. viscosa*) or whole plants (*C. pumilum*) were captured with a digital camera at each sampling time point.

**Analysis of chlorophyll and chlorophyll catabolites**

Chl was extracted from freeze-dried samples in 80% acetone and supernatants were analyzed spectrophotometrically (Strain, Cope and Svec 1971). Phyllobilins were extracted and analyzed by HPLC (setup "1") as previously described (Christ *et al*. 2012), except that freeze-dried samples were homogenized with 18 volumes (w/v; *X. viscosa*) or 50 volumes (w/v; *C. pumilum*) of 50 mM phosphate buffer, pH 7:methanol (1:3, v/v). Co-injection analysis was performed using another HPLC system [setup "2" (Scherl *et al*. 2012); Dionex UltiMate 3000 system (pump and diode array detector)] equipped with a C18 ODS column (5 µm, 250 x 4.6 mm, Phenomenex), which was developed with a gradient (flow rate 0.5 mL $min^{-1}$) of solvent B (100% methanol) in solvent A (10 mM ammonium acetate buffer, pH 7.0) as follows: 38% during 2 min, 38% to 64% in 27 min, 64% to 100% in 3 min

and 100% during 3 min. *Zm*-NCC-2 was prepared from senescent *Z. mays* leaves as described (Berghold *et al*. 2006).

For MS analysis, a *Xv*-NCC-3-containing fraction was isolated by HPLC using setup "2" (see above), which had the following spectroscopic characteristics: UV/Vis: $\lambda_{max}$ = 315 nm. Circular dichroism (CD; c = 3.9 $10^{-5}$ M): $\lambda_{min/max}$ [nm] ($\Delta\varepsilon$) = 316 (4.5), 281 (-4.1), 251 sh (-1.4), 224 (4.9). The sample of *Xv*-NCC-3 was further analyzed by electro-spray ionization mass spectrometry (ESI-MS; Finnigan LCQ classic, positive ion mode, spray voltage 4.25 kV): m/z (% intensity) = 883.2 (14, $[M-H+2K]^+$); 867.2 (12, $[M-H+Na+K]^+$); 845.2 (77, $[M+K]^+$); 829.3 (41, $[M+Na]^+$); 809.2 (12), 808.2 (44), 807.2 (100, $C_{41}H_{51}N_4O_{13}^+$, $[M+H]^+$); 775.3 (33, $[M-CH_4O+H]^+$); 684.2 (21, $[M-C_7H_9NO$ (ring A)$+H]^+$); 652.3 (7, $[M$-ring A$-CH_4O+H]^+$); 645.2 (18, $[M-C_6H_{10}O_5$ (glucose)$+H]^+$); 613.2 (13, $[M-CH_4O$-glucose$+H]^+$).

**Protein extraction and immunoblot analysis**

Total leaf proteins were prepared as described (Ingle, Smith & Sweetlove 2005) with the following modifications. Total proteins were isolated from freeze-dried samples by homogenization in 15 volumes (w/v; *X. viscosa*) or 25 volumes (w/v; *C. pumilum*) of ice-cold extraction buffer [0.5 M Tris–HCl, pH 7.5, 10 mM EDTA, 1% (v/v) Triton X-100, 2% (v/v) 2-mercaptoethanol] complemented with a protease inhibitor cocktail (Complete; Roche Applied Science). Samples were centrifuged at 12'000g for 5 min and protein concentration of the supernatant determined using the Bradford Assay (Bio-Rad). Proteins were subsequently precipitated with chloroform-methanol (Wessel & Flügge 1984) and analyzed by SDS-PAGE and immunoblotting as described (Pružinská *et al*. 2007; Schelbert *et al*. 2009). SDS-PAGE gels were loaded with proteins extracted from equal amounts of dry weight. The following antibodies were used for immunoblot analysis: polyclonal antibodies against LHCb1 and PsbA (1:2000; AgriSera) and monoclonal (*X. viscosa*; 1:500) or polyclonal (*C. pumilum*; 1:2000) antibodies against PAO (kindly provided by Prof. John Gray, University of Toledo; Gray *et al*. 2004).

**RESULTS**

198 **Relative water content, chlorophyll content and leaf morphology**

199 *X. viscosa* plants were dehydrated by withholding irrigation. Leaf RWC consequently dropped to
200 below 10% within 15 d (Fig. 2A). Following 7 d in the desiccated state, plants were irrigated. During
201 this rehydration period, leaf RWC increased rapidly and full turgor was reached after 3 d. Chl was
202 gradually degraded during the dehydration period leading to almost Chl-free dehydrated leaves (Fig.
203 2B). During rehydration, Chl biosynthesis was rapidly induced and concentrations reached 75% of
204 those observed in leaves prior to the water deficit (full turgor, unstressed) within 10 d. Because of the
205 water and Chl loss, leaf morphology changed dramatically during the dehydration and rehydration
206 period (Fig. 2C). Leaves folded along their midribs and changed color from green to yellow due to Chl
207 degradation and unmasking of other leaf pigments. During the recovery period, rehydration preceded
208 Chl resynthesis, and therefore fully rehydrated leaves exhibited a transition phase between yellow and
209 green color.

210 Under comparable dehydration conditions, not all leaves of *C. pumilum* responded consistently
211 (Fig. 3). The youngest leaves ("non-senescent") shrunk and turned green-purple due to the loss of
212 water and the accumulation of anthocyanins, as previously reported (Farrant 2000). In contrast, the
213 oldest leaves ("senescent") changed color from green to yellow and did not shrink as much as "non-
214 senescent" leaves (Fig. 3C). These visual observations corroborated changes in Chl concentrations,
215 which did not significantly change in "non-senescent" leaves but dramatically decreased in
216 "senescent" leaves during dehydration (Fig. 3B). During rehydration, "non-senescent" leaves
217 recovered full turgor (increase of RWC from 5% to 90%, Fig. 3A) and Chl concentrations comparable
218 to those observed in leaves prior to the water deficit (full turgor, unstressed, Fig. 3B) whereas
219 "senescent" leaves did not recover and died.

220

221 **PAO abundance increases during dehydration in *X. viscosa* and *C. pumilum* leaves**

222 Total protein profiles of *X. viscosa* leaves were investigated during dehydration and rehydration
223 by SDS-PAGE and Coomassie blue staining (Fig. 4A). The total protein concentration (μg $mg^{-1}$ DW)
224 did not change significantly during dehydration and, therefore, for each sample the same amount of
225 protein (fifteen microgram) was loaded on SDS-PAGE gels. Levels of large and small subunits of

Rubisco, indicated with black arrowheads in Fig. 4A, stayed rather constant. However, immunoblot analysis revealed changes of the levels of thylakoid proteins involved in photosynthesis as previously reported for poikilochlorophyllous *Xerophyta* species (Fig. 4B; Collett *et al*. 2003; Collett *et al*. 2004; Ingle *et al*. 2007; Ingle *et al*. 2008; Pérez *et al*. 2011). Indeed, PsbA, a subunit of the core complex of PSII, was quantitatively degraded during dehydration and resynthesized during rehydration, similarly to Chl. The same pattern was observed for LHCb1, a component of the light-harvesting antennae of PSII.

To investigate whether Chl is broken down through the well-described PAO/phyllobilin pathway, PAO protein levels were investigated during dehydration and rehydration (Fig. 4B). PAO abundance increased during dehydration, but decreased during rehydration; i.e. it was negatively correlated with the concentration of Chl. Surprisingly, the PAO abundance was highest after 1 d of rehydration.

In contrast to *X. viscosa* in *C. pumilum* leaves, large proportions of proteins were degraded during dehydration and resynthesized during rehydration (Fig. 4C; Bernacchia, Salamini & Bartels 1996). Indeed, protein content decreased by 50% in "non-senescent" leaves and by more than 95% in "senescent" leaves. Therefore, SDS-PAGE gels were loaded based on equal amounts of plant material (dry weight; five milligrams). Abundance of the large and small subunits of Rubisco (indicated with black arrowheads in Fig. 4C) as well as photosystem components such as PsbA and LHCb1 (Fig. 4D) decreased dramatically in "senescent" leaves. Furthermore, Rubisco and photosystem subunits appeared to be partially degraded in "non-senescent" leaves (Fig. 4C and D), which is in agreement with transcript accumulation patterns (Bernacchia *et al*. 1996). PAO abundance increased in both "senescent" and "non-senescent" leaves, suggesting that chlorophyll degradation occurs in the entire plant during desiccation (Fig. 4D).

**Leaves of *X. viscosa* and *C. pumilum* accumulate phyllobilins during dehydration**

The water deficit-induced increase in PAO abundance in leaves of *X. viscosa* points to a possible involvement of the PAO/phyllobilin pathway in the breakdown of Chl. PAO is a key enzyme of the pathway because it opens the macrocycle of pheophorbide *a* and therefore determines the basic structure of all downstream linear catabolites (phyllobilins; Kräutler & Hörtensteiner 2013). In order

to investigate the presence of such catabolites, samples were collected at different time points during dehydration and rehydration and analyzed by reversed-phase HPLC (Fig. 5A). Spectral analysis of the resulting chromatograms allowed the identification of four fractions, tentatively named *Xv*-NCC-1 to -4, showing typical NCC absorbance properties (Fig. 5B, Supplemental Fig. S1; Kräutler & Hörtensteiner 2013). These fractions were absent prior to dehydration and increasingly accumulated in the leaves during dehydration and Chl degradation. After 10 d of subsequent rehydration, they had almost completely disappeared. In-depth analysis of the major fraction, *Xv*-NCC-3, by mass spectrometry revealed that this NCC has the same mass (m/z $[M+H^+]$ = 807.2; Fig. 5B) as *Zm*-NCC-2, an NCC described from *Zea mays* (Berghold *et al*. 2006). HPLC analysis of purified fractions showed that *Xv*-NCC-3 and *Zm*-NCC-2 exhibited the same retention time when analyzed independently and co-eluted as a single fraction when co-injected as a 1:1 (w/w) mixture (Fig. 5C). This observation indicates that *Xv*-NCC-3 has the same structure as *Zm*-NCC-2 (Fig. 5D; Berghold *et al*. 2006). *Xv*-NCC-3/*Zm*-NCC-2 carries a methyl ester group at $C13^2$ position (see Fig. 1) indicating the likely absence in *X. viscosa* of a homolog of METHYLESTERASE16, responsible for the ester hydrolysis in *A. thaliana* (Christ *et al*. 2012). The $C8^2$-ethyl moiety of *Xv*-NCC-3 is hydroxylated and subsequently glycosylated (see Fig. 5D); respective modifications are also known to occur in natural NCCs from e.g. *A. thaliana, Brassica napus, Nicotiana rustica, Pyrus communis* and *Z. mays* (Kräutler & Hörtensteiner 2013). The structural identity of *Xv*-NCC-3 with *Zm*-NCC-2 also implied the same stereoselectivity of the respective RCCRs from *X. viscosa* and *Z. mays* (Berghold *et al*. 2006). RCCR reduces red Chl catabolite (RCC) produced by PAO to *p*FCC (Pružinská *et al*. 2005; Pružinská *et al*. 2007). Depending on the plant species, RCCRs exhibit different stereospecificities in the reduction of the C20/C1 (see Figs. 1 and 5D) double bond of RCC and therefore yield one of the two possible C1-stereoisomers, i.e. *p*FCC or *epi-p*FCC (Moser *et al*. 2009; Kräutler & Hörtensteiner 2013). From the identity of *Xv*-NCC-3 and *Zm*-NCC-2, the C1 stereochemistry of *X. viscosa* RCCR can be deduced: it yields *epi-p*FCC like the RCCRs from *Z. mays*, *N. rustica*, *Spinacia oleracea* and *Cercidiphyllum japonicum* (Kräutler & Hörtensteiner 2013).

Like observed for *X. viscosa*, dehydration of *C. pumilum* also led to the accumulation of Chl catabolites in leaves (Fig. 6A and B). In “senescent” leaves, in which the Chl content decreased

dramatically during desiccation (Fig. 3B), several NCCs and YCCs were found and were classified on the basis of their characteristic UV/Vis spectra (Fig. 6A, Supplemental Fig. S1; see Moser *et al*. 2008; Ulrich *et al*. 2011). However, phyllobilins were also identified in "non-senescent" leaves, indicating that Chl is also partially degraded in these leaves, although the decrease in Chl content was not significant when determining Chl concentrations at the different sampling times (Fig. 3B). The accumulation of NCCs and YCCs (Fig. 6A and B), together with the increase in PAO abundance (Fig. 4D), suggests that water deficit-induced Chl breakdown also occurs in *C. pumilum* and is mediated enzymatically by the PAO/phyllobilin pathway. During rehydration of "non-senescent" leaves, colorless Chl catabolites disappeared rapidly (Fig. 6A and B), comparable to the observation in *X. viscosa* (Fig. 5A).

## DISCUSSION

The ultimate fate of a senescing leaf is death; however, leaf senescence can be considered to be reversible until a "point of no return", indicating that the processes of leaf senescence themselves take place in living tissue (Thomas *et al*. 2003; Thomas 2013). Indeed, chloroplast-to-gerontoplast (senescent chloroplast) transition has been shown to be reversible in *N. rustica* (Zavaleta-Mancera *et al*. 1999a; Zavaleta-Mancera *et al*. 1999b). Shoot decapitation of senescing *N. rustica* plants leads to leaf regreening, an effect that is enhanced by treatment with cytokinins, which are known to inhibit senescence (Gan & Amasino 1997). Leaf regreening was shown to involve resynthesis of functional thylakoid membranes within gerontoplasts rather than *de novo* formation of chloroplasts (Zavaleta-Mancera *et al*. 1999a, b).

In this study, we focused on the well characterised leaf de- and regreening associated with Chl breakdown in poikilochlorophyllous resurrection plants (Tuba *et al*. 1996; Sherwin & Farrant 1998; Farrant 2000), focusing on *X. viscosa*. We hypothesized that similar reversible mechanisms of chloroplast dismantling are shared between age-dependent leaf senescence of higher plants and leaf dehydration of poikilochlorophyllous plants. Although, the process of poikilochlorophylly (complete loss of Chl during desiccation of resurrection plants) has been well described (Tuba *et al*. 1994;

Sherwin & Farrant 1996; Tuba *et al*. 1998), Chl breakdown has been speculated to occur via nonenzymatic photooxidation events (Tuba *et al*. 1996; Proctor & Tuba 2002). Likewise, senescence processes of resurrection plants, including Chl breakdown, are poorly understood (Denev, Stefanov & Terashima 2012) and an involvement of the PAO/phyllobilin pathway has not been demonstrated. Here, we provide evidence that during dehydration of *X. viscosa* Chl is degraded enzymatically via the PAO/phyllobilin pathway (Kräutler & Hörtensteiner 2013) to various NCCs (Fig. 5), the major one (*Xv*-NCC-3) being identical to *Zm*-NCC-2 of maize (Berghold *et al*. 2006). Although we were unable to precisely quantify the amounts of phyllobilins during desiccation, leaving open the possibility that a minor fraction of Chl may be degraded non-enzymatically, the increases in phyllobilin and PAO abundance during dehydration (Fig. 4B), suggest that water deficit-induced poikilochlorophylly in *X. viscosa* leaves is mechanistically comparable to the highly regulated PAO/phyllobilin pathway active during leaf senescence and fruit ripening (Kräutler & Hörtensteiner 2013).

A major function of Chl breakdown in leaves and fruits is the detoxification of photoactive Chl and breakdown intermediates, thereby increasing cell viability during senescence. Indeed, suppression of PAO results in a premature cell death phenotype, which is caused by the accumulation of phototoxic pheophorbide *a* (Pružinská *et al*. 2003; Tanaka *et al*. 2003). As a consequence, respective mutants such as *A. thaliana pao1* and *Z. mays lls1* exhibit severe defects in growth, senescence and fertility (Gray *et al*. 1997; Pružinská *et al*. 2005). Similarly, it has been argued that Chl degradation in poikilochlorophyllous resurrection plants is a strategy to avoid photooxidative damage caused by Chl in dry leaves, and, thus, enhances plant survival during and after dehydration (Proctor & Tuba 2002). It is tempting to speculate that in poikilochlorophyllous species mutations of components of the PAO/phyllobilin pathway, which during senescence of desiccation-intolerant plants causes the retention of Chl within Chl-apoprotein complexes and/or the accumulation of photodynamic breakdown intermediates (Pružinská *et al*. 2005; Park *et al*. 2007; Pružinská *et al*. 2007; Hörtensteiner 2009), would cause photooxidative damage during desiccation, and, thus, would dramatically decrease the resurrection capacity of these plants.

By contrast, homoiochlorophyllous resurrection species retain large proportions of Chl during desiccation. Such plants have developed other protection mechanisms such as leaf curling which, apart

from chemical protection mechanisms, has an important light shading effect on Chl-apoprotein complexes (Farrant *et al*. 2003). Nevertheless, homoiochlorophyllous species also degrade (some) Chl during desiccation (Farrant *et al*. 1999; Alamillo & Bartels 2001). We could demonstrate that a comparable enzymatic mechanism of Chl breakdown (i.e. the PAO/phyllobilin pathway) is also active in the leaves of *C. pumilum*. A major difference was found in the type of phyllobilins that accumulate during dehydration. The specific occurrence of YCCs, i.e. oxidation products of NCCs that may be formed in vivo (Moser *et al*. 2008; Ulrich *et al*. 2011, Kräutler & Hörtensteiner 2013) in *C. pumilum* leaves suggests increased oxidative stress potential in this homoiochlorophyllous species. Interestingly, YCC accumulation was not limited to the surviving leaves ("non-senescent") of the plant, but also occurred in leaves that did not recover from dehydration ("senescent"). This finding may indicate that, during dehydration, leaves of homoiochlorophyllous species are subjected to more severe oxidative stress compared to those of poikilochlorophyllous species, presumably due to the higher concentrations of Chl present in the former. In line with this, homoiochlorophyllous species have been shown to accumulate large amounts of malondialdehyde during desiccation (Djilianov *et al.* 2011; Jovanovic *et al.* 2011). Malondialdehyde is a breakdown product of lipid peroxidation and, thus, an indicator of cellular oxidative damage.

For many desiccation-intolerant species phyllobilins have been shown to progressively accumulate in the vacuole during senescence and to persist until the leaf dies or sheds (Kräutler & Hörtensteiner 2013). Interestingly, in both *X. viscosa and C. pumilum*, rehydration was accompanied by the disappearance of accumulated phyllobilins. The mechanism of this phenomenon remains unknown, but likely occurs through further degradation of the tetrapyrrole backbone of the phyllobilins. In both homoio- and poikilochlorophyllous species, the central vacuole is fragmented to small vacuoles during dehydration and re-formation of the large central vacuole (during rehydration) is thought to occur by fusion of these smaller ones (Rascio & Rocca 2005). Thus, we assume that phyllobilin degradation should occur during this vacuolar merging phase.

Parallel to Chl breakdown, PS core and antennae subunits are also degraded during dehydration of *X. viscosa* (Fig. 4B). Both Chl biosynthesis and degradation are known to be linked with PS assembly and disassembly, because Chl molecules are intimately integrated within PS subunits

(Nelson & Yocum 2006; Hörtensteiner 2009; Tanaka & Tanaka 2011). Consequently, suppression of genes involved in the early steps of the PAO/phyllobilin pathway inhibits the degradation of several PS components, in particular LHCII subunits (Park *et al*. 2007; Schelbert *et al*. 2009). In addition to its detoxifying role, Chl degradation during leaf senescence is thought to be a prerequisite for the degradation and remobilization of PS proteins, which contain a significant fraction of nitrogen present in mesophyll cells (Hörtensteiner & Feller 2002). From this perspective, the correlation between the degradation of Chl and Chl-binding proteins observed in both the homoio- and poikilochlorophyllous species is not overly surprising. By contrast, however, Rubisco was not degraded during dehydration of *X. viscosa* (Fig. 3A), pointing to a major difference between leaf desiccation and senescence. Previous observations have noted that the mechanisms of chloroplast dismantling during dehydration of poikilochlorophyllous plants differs from leaf senescence (Proctor & Tuba 2002) and that senescence reduces desiccation tolerance (Gaff 1986). During leaf senescence, Rubisco, which accounts for 20–30% of total leaf nitrogen, is also degraded for nitrogen remobilization (Feller, Anders & Mae 2008). It is most likely that nitrogen remobilization is irrelevant during dehydration of resurrection plants. Instead, it is reasonable to assume that the selective degradation of Chl-binding proteins during dehydration in *X. viscosa* is a prerequisite to enable Chl degradation (preventing photooxidation) with the converse being true during leaf senescence (Chl broken down as a prerequisite for nitrogen remobilization of Chl-binding proteins). In this respect, the processes occurring in *C. pumilum*, i.e. patterns of both Rubisco and PS subunit degradation correlating with Chl degradation (Fig. 4), resemble leaf senescence. It will be interesting to elucidate to what extent this assumption can be extrapolated to other aspects of senescence, for example by comparative analysis of senescence-associated gene expression in homoio- and poikilochlorophyllous species.

The finding that the PAO/phyllobilin pathway is involved during leaf desiccation in resurrection plants indicates that the same mechanism of Chl breakdown is used during leaf desiccation and leaf senescence, although the main goals of these two processes are different (survival and nutrient remobilization, respectively). Even desiccation-intolerant angiosperms exhibit desiccation tolerance, which, however, is restricted to seeds. Seed desiccation enables protection of the embryo in a dormant state until conditions are favorable for germination (Finkelstein *et al*. 2008). Seed maturation, similar

394 to leaf desiccation in resurrection plants (Rascio & Rocca 2005; Illing *et al*. 2005), involves the
395 accumulation of considerable quantities of non-reducing di- and oligosaccharides, compatible solutes
396 and specific proteins such as late embryogenesis abundant proteins and heat shock proteins (Hoekstra,
397 Golovina & Buitink 2001). During seed maturation, the embryo degrades Chl and, interestingly, Chl
398 degradation seems to also follow the PAO/phyllobilin pathway. Thus, when *B. napus* or *A. thaliana*
399 plants are exposed to freezing temperatures during seed maturation, seed Chl breakdown is blocked
400 (Chung *et al*. 2006; Delmas *et al*. 2013). *PAO* expression was shown to be reduced under such
401 conditions and pheophorbide *a* accumulated in frost-exposed seeds (Chung *et al*. 2006). Likewise,
402 absence of STAY-GREEN, which is essential for Chl breakdown in senescing leaves (Hörtensteiner
403 2009), also causes retention of Chl during seed maturation. One famous example of mutants deficient
404 in STAY-GREEN is Mendel's green cotyledon mutant (Armstead *et al*. 2007). The capacity of
405 embryo degreening seems to correlate with seed storability, and germination rates of mature seeds
406 have been demonstrated to be inversely proportional to the Chl level (Jalink *et al*. 1998). Thus,
407 suppression of Chl *b* to *a* conversion in *A. thaliana* leads to Chl retention in the embryo during seed
408 drying and dramatically decreases seed germination capacity (Nakajima *et al*. 2012).

409 In summary, we have provided evidence that both homoio- and poikilochlorophyllous plants
410 degrade Chl via the PAO/phyllobilin pathway. Our findings have extended the physiological
411 conditions under which this pathway is active to now include the leaves of resurrection plants during
412 dehydration. Our data support the notion that the PAO/phyllobilin pathway of Chl breakdown is not
413 restricted to conditions that ultimately lead to the death of degreening tissues, like senescing leaves
414 and ripening fruits, but also occurs in tissues that naturally regreen at later stages, like during (epigeal)
415 seed germination and rehydration of leaves of poikilochlorophyllous resurrection plants.

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## FIGURE LEGENDS

**Figure 1.** Structural outline of the PAO/phyllobilin pathway of Chl breakdown. The chemical constitutions of Chl (Chl *a*, $R^0 = CH_3$; Chl *b*, $R^0 = CHO$) and of selected Chl catabolites are shown. Relevant carbon atoms are labeled in *p*FCC. Sites of peripheral modifications as present in DNCCs and NCCs identified from different plant species (Kräutler & Hörtensteiner 2013) are indicated ($R^1$ – $R^3$). YCCs have been shown to be derived from respective NCCs by oxidation (Moser *et al*. 2008). The reactions of PAO and RCCR are indicated. Note that depending on the source of RCCR two C1 stereoisomers of *p*FCC, *p*FCC or *epi*-*p*FCC, can be formed. Abbreviations are as indicated in the text.

**Figure 2.** RWC (a), Chl (b) content and leaf morphology (c) of *X. viscosa* leaves during dehydration (-$H_2O$) and rehydration (+$H_2O$). Data are mean values of a representative experiment with three

666 replicates. Error bars indicate SD. In (c), leaf morphology and color during dehydration and
667 rehydration were imaged using a digital camera. Bar = 0.5 cm.
668
669 **Figure 3.** RWC (a), Chl (b) content and plant morphology (c) of *C. pumilum* leaves during
670 dehydration (-$H_2O$) and rehydration (+$H_2O$). Four time points were assessed: "hydrated" (0 d),
671 "partially dehydrated" (10 d), "dried" (15 d) and "rehydrated" (+ 7 d). Triangles are used to display
672 results obtained from leaves that survived the experiment ("non-senescent") and squares refer to leaves
673 that turned yellow and did not survive the experiment ("senescent"). Data are mean values of a
674 representative experiment with three biological replicates. Error bars indicate SD. In (b), the
675 significance of differences between "non-senescent" samples was determined using a two tailed t-test.
676 In (c), leaf morphology and color during dehydration and rehydration were imaged using a digital
677 camera. Bar = 2.0 cm.
678
679 **Figure 4.** Protein analyses in *X. viscosa* (a and b) and *C. pumilum* (c and d) leaves during dehydration
680 and rehydration. (a) and (c) Total leaf protein samples corresponding to 5 mg of dry weight were
681 separated by SDS-PAGE and stained with Coomassie blue. Rubisco (large and small subunits) are
682 indicated with black arrowheads. (b) and (d) Immunoblot analysis of PsbA, LHCb1 and PAO levels.
683 After SDS-PAGE as shown in panel (a) and (c), proteins were transferred onto a nitrocellulose
684 membrane and visualized by immunoblotting using anti-PsbA, -LHCb1 and -PAO antibodies. For
685 more details, see "Materials and Methods". Standard protein masses are given in kDa on the right.
686
687 **Figure 5.** Phyllobilin analyses in *X. viscosa* leaves during dehydration and rehydration. (a) Catabolites
688 were separated using the HPLC setup “1” described in "Materials and Methods". $A_{254}$ are shown. For
689 clarity, only the relevant parts of the HPLC traces are shown. (a) Mass and UV/Vis spectra of *Xv*-
690 NCC-3. For more details on MS data of *Xv*-NCC-3, see "Materials and Methods". A UV/Vis spectrum
691 of *Xv*-NCC-3 is shown in the inset. (c) HPLC co-injection of *Xv*-NCC-3 and *Zm*-NCC-2. Note that this
692 experiment was performed with the HPLC setup “2” described in "Materials and Methods" and

693 indicates that the two NCCs have the identical constitution. $A_{320}$ are shown. (d) Chemical structure of
694 *Xv*-NCC-3/*Zm*-NCC-2. Relevant atoms are labeled.
695
696 **Figure 6.** Analysis of phyllobilins in *C. pumilum* leaves during dehydration and rehydration. (a)
697 Catabolites were separated using the HPLC setup "1" described in "Materials and Methods". $A_{254}$ are
698 shown. For clarity, only the relevant parts of the HPLC traces are shown. (b) Relative amounts of
699 NCCs and YCCs in "non-senescent" and "senescent" leaves of *C. pumilum* during dehydration and
700 rehydration. Data are mean values of a representative experiment with three replicates and are based
701 on equal leaf dry weight. Error bars indicate SD.
702
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704 **SUPPORTING INFORMATION**
705 Additional Supporting Information may be found in the online version of this article:
706 **Figure S1.** UV/Vis spectra of phyllobilins identified in this study.
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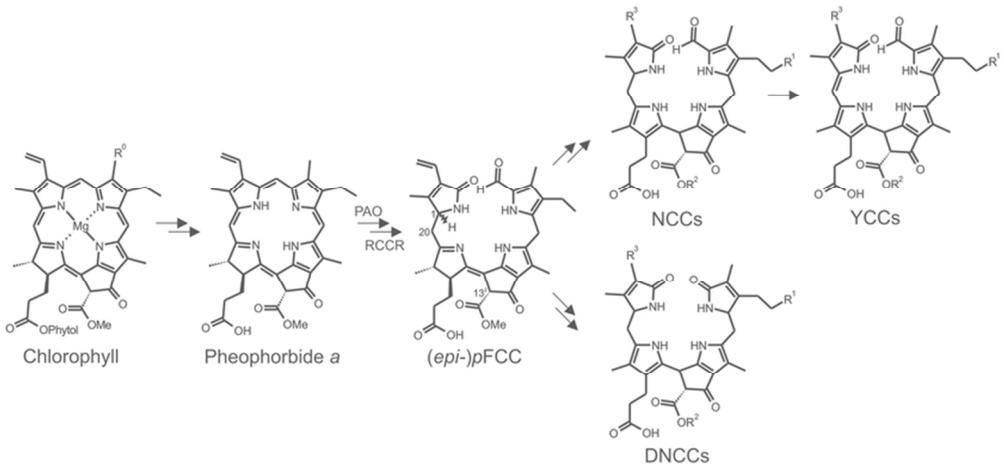
708

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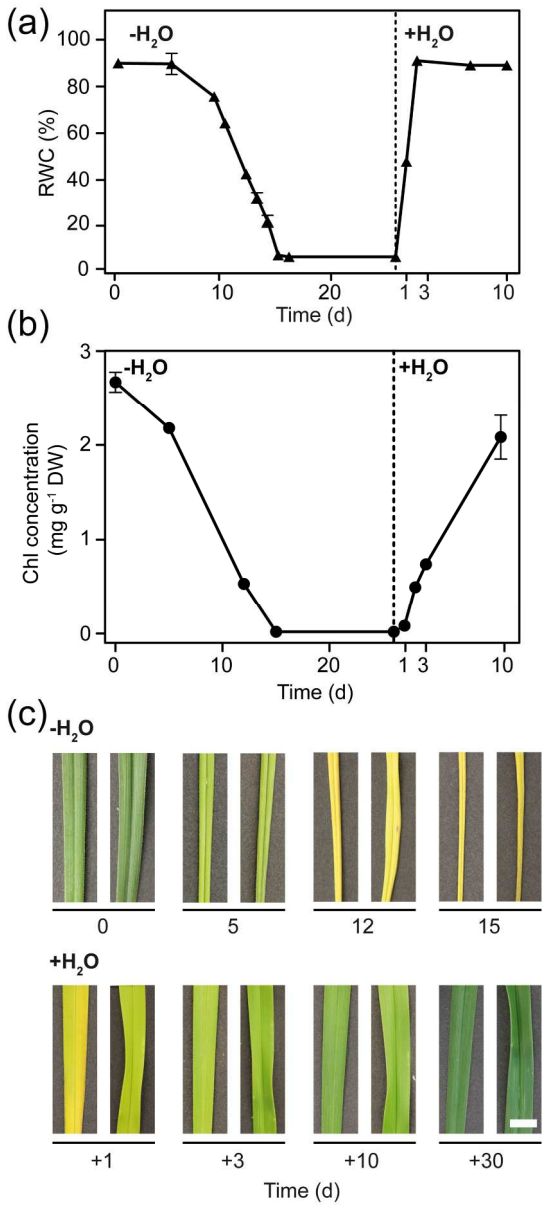


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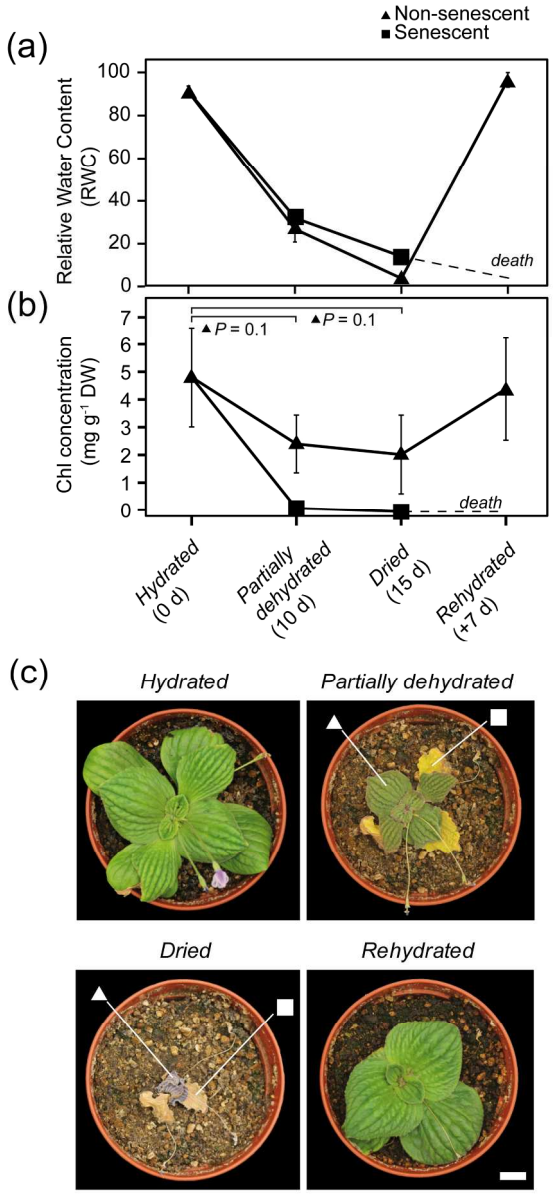


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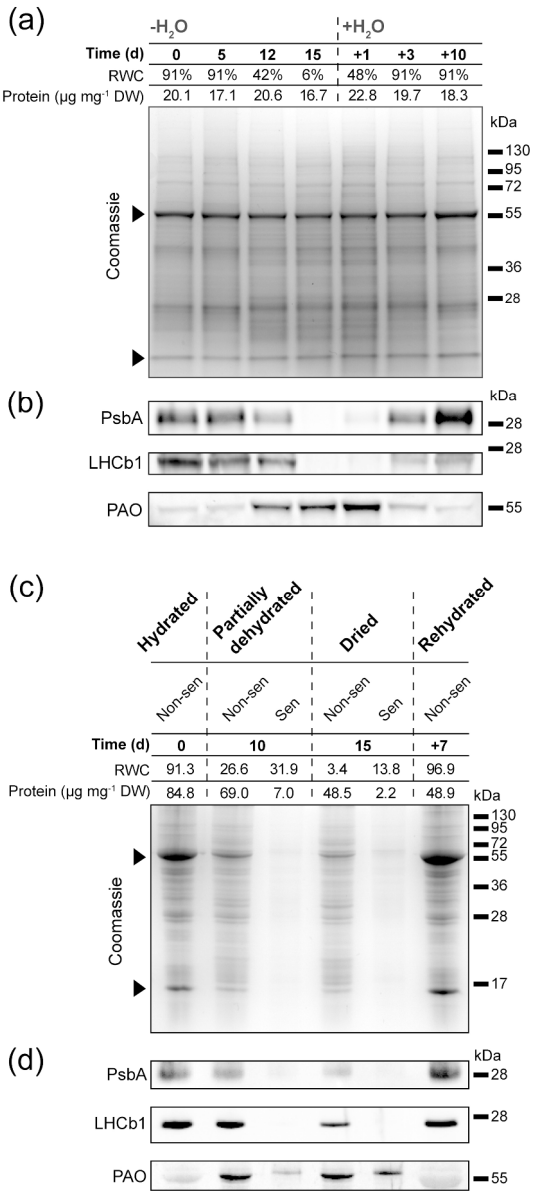


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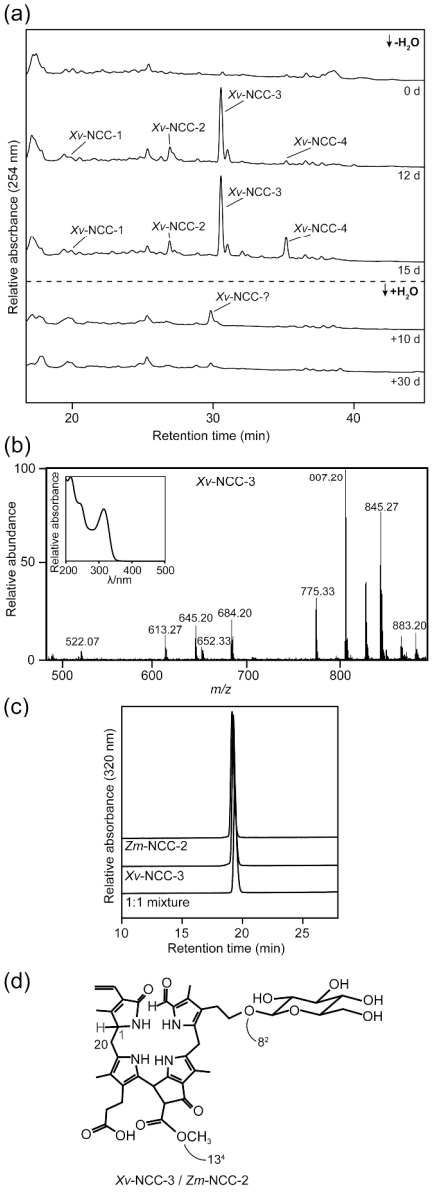


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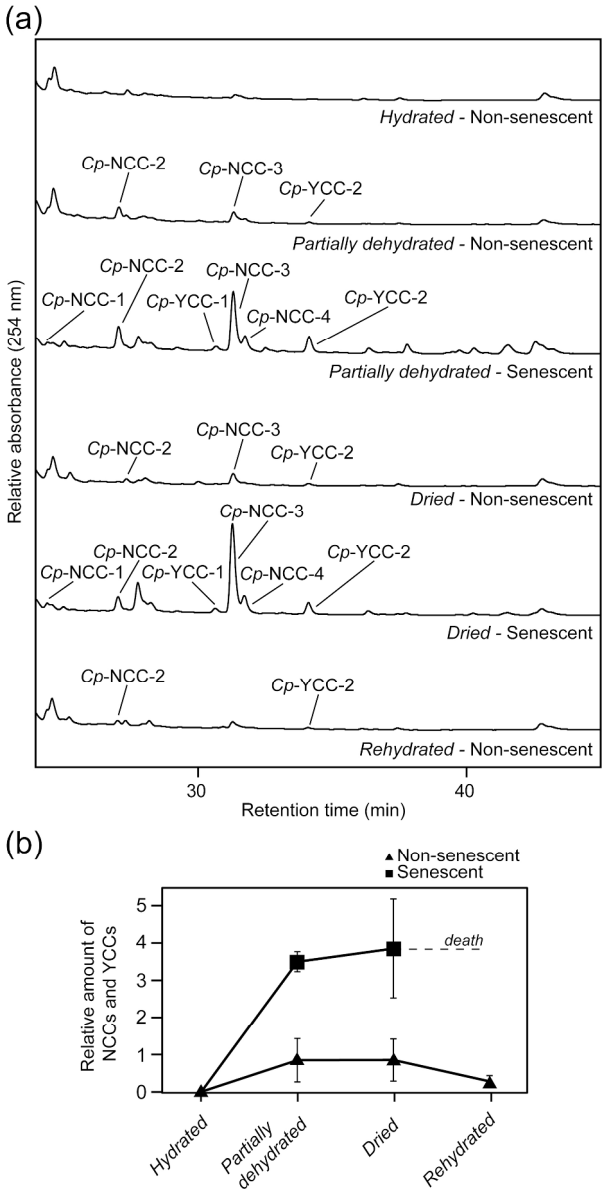


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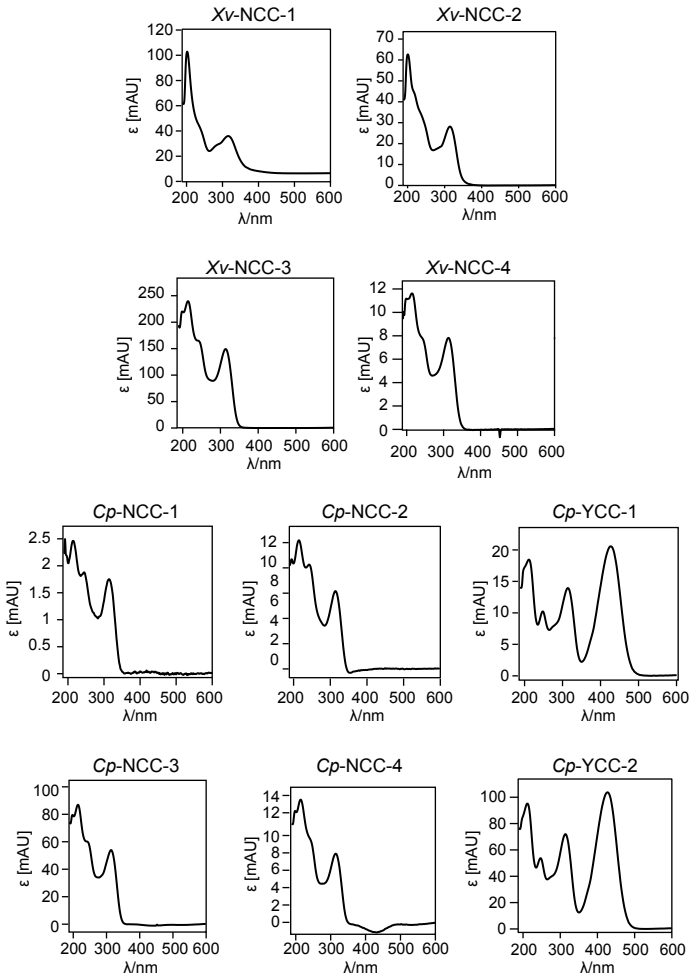


**Figure S1.** UV/Vis spectra of phyllobilins identified in this study